Environmental Health Perspectives
Vol. 93, 45–49, 1991

Oncogenes and Radiation Carcinogenesis

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Current research indicates a role for several oncogenes in radiation-induced carcinogenesis *in vivo* and cell transformation *in vitro*. Certain oncogenes are probably also involved in some cases of human cancer caused by exposure to nonionizing radiation and may play a mechanistic role in the phenomenon of radioresistance seen in later stages of tumor progression. The mechanisms of oncogene activation seen in radiation-induced tumors include point mutations, gene amplification, and changes in gene expression. Genetic factors associated with target species, strain, and tissue type play an important role in determining the specific nature of oncogene activation by radiation exposure. Using the rat skin as a model for cancer induction by ionizing radiation, we found concurrent activation of K-*ras* and c-*myc* oncogenes in end-stage tumors. Amplification of the *myc* gene proved to occur during a late stage of tumor progression and is not an early initiating event resulting from the direct action of radiation on target cells. The importance of tissue specificity, tumor cell heterogeneity, and physical characteristics of the radiation exposure are discussed.

Introduction

Ionizing radiation is a well-established environmental carcinogen for humans and animals. Over several decades a large body of data has been generated to characterize the effects of dose, dose rate, linear energy transfer, age, and other factors on cancer induction in experimental models and human populations exposed to high levels of radiation (*1*). Studies of carcinogenic mechanisms have shown that ionizing radiation produces a variety of genetic lesions, including chromosomal aberrations, DNA strand breaks, deletions, and gene rearrangements (*2*). More recently, point mutations have been reported to be a consequence of radiation exposure (*3*).

The hypothesis that cellular oncogenes represent an important class of target genes for the action of chemical carcinogens has been strongly supported by data from many laboratories using several model systems (*4–9*). Considering the well-known effects of radiation as a DNA damaging agent, it is logical to ask whether there is evidence that specific cellular genes might represent target sequences for the action of ionizing radiation related to carcinogenesis. Such sequences could include oncogenes, tumor-suppressor genes, integrated viral sequences, or other genes. In recent years a number of laboratories have published results of research on the interaction between radiation and cellular oncogenes.

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Ras Gene Activation

Evidence that cellular oncogenes (both known and yet to be characterized) are in fact likely to be targets for the effects of ionizing radiation has been obtained using approaches similar to those used earlier and more extensively for chemical carcinogens. For example, the first demonstration of an activated *ras* oncogene in radiation-induced tumors was done by transfection of mouse thymoma DNA into NIH 3T3 cells (*10*). This group found that the K-*ras* gene was specifically activated in radiation-induced thymomas of (AKR × RF/J) F1 mice, while N-*ras* was activated in chemically induced tumors.

The K-*ras* oncogene has been specifically implicated in other model systems of radiation-induced carcinogenesis. In 50% of rat skin carcinomas (*11*) and 60% of rat thyroid tumors (*12*), activated K-*ras* genes were detected. In the latter case, the activation of K-*ras* was also carcinogen-specific, since nitrosomethylurea (NMU)-induced tumors of the same type contained exclusively H-*ras* (*12*).

However, K-*ras* is not the only specific target gene for ionizing radiation. Species and strain genetic factors are important in determining which (if any) oncogene will be part of the transformation pathway. This was shown most notably by Pellicer and co-workers, who found that in radiation-induced thymomas from RF/J mice, both K- and N-*ras* genes were mutated, while in C57Bl/6J mice, these tumors contained mostly N-*ras* and novel non-*ras* oncogenes (*13,14*). In canine leukemia induced by exposure to gamma radiation from a cobalt source, the N-*ras* oncogene was found to be activated (*15*). A murine osteosarcoma cell line derived from a ^{90}Sr-induced tumor contained activated H-*ras* (*16*).

The examples discussed so far have been from animal model studies using ionizing radiation. In humans, ultraviolet radiation is an important environmental carcinogen, especially in rare DNA repair-deficiency syndromes such as xeroderma pigmentosum (XP). In two tumors isolated from a single XP patient, the N-*ras* oncogene was found to be mutationally activated (*17*). In addition, amplification of the c-*myc* and H-*ras* oncogenes was detected. The mutational activation of the N-*ras* gene was a rare event, since tumors from seven other patients revealed no mutations in *ras* genes. However, six of ten tumors tested did exhibit amplified H-*ras* (*17*). Another laboratory has reported that a melanoma cell line established from an XP patient also contained an N-*ras* oncogene activated by the same codon 61 point mutation seen in the first study (*18*).

In a survey of human skin squamous cell carcinomas excised from sun-exposed body sites, it was reported that four of the eight tumors examined exhibited activated H-*ras*, and two contained novel non-*ras* genes (*19*). The specificity for N-*ras* activation in the two XP studies is not likely therefore to be a function of carcinogen-specific (UV) or tissue-specific (human skin) phenomena, but may possibly be related to the XP genotype.

Other Oncogenes

As might be predicted from the diverse effects of radiation on the genome, point mutational activation of *ras* genes is only one of several types of oncogene alterations seen in radiation-induced transformation. We have shown that many of the large rat skin tumors that harbor an active K-*ras* gene also exhibit amplified and overexpressed c-*myc* (*11*). Overexpression and amplification of the c-*myc* gene was also seen in a number of mouse tumor types induced by ionizing radiation (*20*). Overexpression of c-*myc* was also observed in some but not all mouse osteosarcomas (*21*) and mouse thymomas (*22,23*).

A number of studies have been published dealing with effects of radiation on oncogenes in cultured cells. The incorporation of ^{125}I into the DNA of Chinese hamster embryo cells led to selective amplification of K-*ras* and H-*ras* (*24*). Ultraviolet radiation, along with other DNA-damaging agents, was found to induce c-*fos* expression in Chinese hamster ovary cells (*25*). The importance of the genetic background of the target cell is again illustrated by the fact that in mouse C3H10T 1/2 cells, UV-induced transformant showed a decreased level of c-*fos* expression, while c-*myc* expression was elevated (*26*). Two laboratories have reported the detection of novel non-*ras* oncogenes capable of producing foci in the NIH 3T3 transfection assay in C3H10T 1/2 cells transformed by X-rays (*27,28*). In one recent study, the question of whether a *ras* gene can serve as a target for activation by radiation was tested directly (*29*). A plasmid containing the proto-N-*ras* oncogene was exposed to UV light and was then capable of transforming Rat 2 cells. The transformants all showed activating mutations at codons 12 or 61 of the transfected N-*ras* gene.

Oncogenes and Radioresistance

An understanding of the role of oncogenes in radiation carcinogenesis is still at an early stage despite the increased attention being devoted to this issue in the last 2 years. Like many chemicals, radiation is a carcinogen that is also an effective therapeutic agent. The killing of cancer cells by radiation is a well-known and important therapeutic modality. Also well known is the phenomenon of resistance to radiation-induced cell killing that often occurs during progression of malignant tumors. Several laboratories have obtained evidence that cellular oncogenes are involved in the development of the radioresistant phenotype. A radiation-resistant human laryngeal cancer cell line was found to contain an activated *raf* oncogene (*30*). The same group has presented evidence that *raf* may be involved in conferring radioresistance since cDNAs for antisense *raf* resulted in modulation of resistance (*31*). Another laboratory found a *raf* gene to be involved in the transforming and radioresistance activities of DNA from a human skin cell line (*32,33*). However, transfection of *raf* and other oncogenes into NIH 3T3 cells produced no clear alterations in radiation sensitivity (*34*). Different results have been reported in a study that showed enhanced radiation resistance in NIH 3T3 cells transformed by transfection with *ras* oncogenes (*35*). Transfection of *ras* and *myc* also conferred an increase in radioresistance to primary fibroblast cells (*36,37*).

The studies of oncogenes and radiation resistance concern a later stage of neoplastic progression than initiation of the transformed phenotype. The issue of timing of oncogene activation during the many temporal stages of carcinogenesis is complex and poorly understood. In many model systems, oncogene activation appears to be an early, perhaps initiating event (*4,38*). One report showing increased sensitivity of C3H10T 1/2 cells to transformation by ionizing radiation after transfection with a c-*myc* gene (*39*) also suggests an early role for c-*myc* expression in radiation-induced cancer.

Rat Skin Carcinogenesis

Tumors of the rat skin have proven to be an excellent model for studying the biological, physical, and molecular aspects of radiation carcinogenesis (*40,41*). Our initial experiments using rat skin tumors induced by ionizing radiation seemed to implicate activation of c-*myc* as a frequent, possibly early event in the carcinogenic process (*11*). However, our more recent data contradicts this idea and instead supports the hypothesis that c-*myc* amplification is associated with a late stage of progression in radiation-induced rat skin carcinogenesis (*42,43*).

There are few experimental models of tumor progression that are as potentially useful for studies of molecular mechanisms as tumors of the skin. The mouse

skin model, one of the most extensively studied, includes clearly defined stages of neoplastic progression from benign papilloma to malignant carcinoma. Although rat skin tumors do not usually arise from benign lesions, this model also has many advantages for the study of progression (*44*). Tumors are visible at an early stage, the growth of individual tumors can be easily followed, tumors can be repeatedly biopsied during growth, and tumors of various histologic types are produced for comparison and analysis of cell-type-specific phenomena.

Tumors were induced by exposure of 4-week-old male rats to doses of 8 to 16 Gy of 0.8 MeV electron radiation. Southern blot hybridization of tumor DNAs to a *c-myc* probe showed gene amplification (5- to 10-fold) and restriction-fragment polymorphisms in 10 of the 12 tumors. Analyses of tumor RNA revealed enhanced c-*myc* expression in amplification-positive tumors. Six of the 12 radiation-induced tumor DNAs tested were positive in the NIH 3T3 focus assay, and Southern blot analysis of transfectant DNA showed that each tumor contained an activated K-*ras* oncogene (*11*). Concurrent activation of both K-*ras* and *c-myc* oncogenes was found in three poorly differentiated clear cell carcinomas. In four of the five well-differentiated squamous cell carcinomas examined, only *myc* activation was seen (*11*).

The frequent activation of the c-*myc* oncogene by amplification and rearrangements is consistent with the known clastogenic effects of ionizing radiation on target DNA. We therefore set out to test the hypothesis that radiation exposure leads directly to activation of c-*myc*, which would be the first step in radiation carcinogenesis. We proceeded therefore to examine the activation of c-*myc* as a function of tumor progression, from first appearance of very small tumors to the development of large rapidly growing malignant cancers.

Approximately 60 samples of rat skin tumors from 0.1 to 20 cm^3 in size were analyzed by Southern blot hybridization for *c-myc* gene copy number. These tumors were mostly epithelial, including squamous cell carcinomas, clear cell carcinomas, basal cell carcinomas, keratosebaceous tumors, and carcinomas *in situ*. Nine sarcoma samples were also analyzed. Increased c-*myc* gene copy number was associated with increased size in the epithelial tumors. There was a progressive increase in the fraction of tumors containing amplified c-*myc* in larger size categories. For example, 7% of squamous cell carcinomas that were less than 1 cm^3 in size exhibited a c-*myc* copy number of 3 or higher, while 47% of these tumors between 1 and 10 cm^3 contained amplified c-*myc*. A similar trend was observed for other epithelial tumors. All six of the epithelial tumors in the size class greater than 10 cm^3 showed amplification of c-*myc*; however, c-*myc* amplification was not detected in any sarcomas regardless of size class. The difference between epithelial tumors and sarcomas was statistically significant ($\chi^2 = 5.9$, $p < 0.01$) and suggests cell-type-specific differences in molecular mechanisms of neoplasia in rat skin. The average degree of c-*myc* amplification also increased in larger tumors from an average of 3-fold in tumors < 1 cm^3 to 6-fold for tumors between 1 and 10 cm^3 and 11-fold for tumors greater than 10 cm^3 in size.

Gene amplification was specific for c-*myc* among the six oncogenes examined. Although amplification of K-*ras*, H-*ras*, *abl*, and *fos* occurred in from 5 to 29% of the epithelial tumors, no correlation was seen with tumor size. In contrast, linear regression analysis of c-*myc* gene copy number with size revealed a highly significant ($R = 0.66$, $n = 50$, $p < 0.001$) correlation between these parameters. Amplification of N-*myc* was never found.

Biopsy Studies

We were able to perform molecular analyses on serial biopsies of growing skin tumors, allowing us to determine the c-*myc* gene copy number as a function of time in individual tumors. Biopsies were performed on 11 tumors from two to five times each. In all but two cases the c-*myc* gene copy number increased in the later biopsies compared to earlier ones. One of the two exceptions was a sarcoma. The other exception was a squamous cell carcinoma that showed a complex pattern of growth and *c-myc* copy number with time. This tumor grew larger with time until the third biopsy, at which point it began to regress. Interestingly, the c-*myc* copy number also first increased and then decreased in the tumor during regression, in parallel with the change in tumor size. A plot of tumor growth rate at each biopsy versus the c-*myc* copy number exhibited a good correlation ($R = 0.95$, $p < 0.05$) between these parameters. The intercept (corresponding to a growth rate of 0) of this curve was very close to 1.

Myc Amplification and Tumor Growth

Since tumor regression in rat skin carcinogenesis is rare, one possible explanation for these results was that the biopsy procedure involving surgical removal of two-thirds of the tumor mass was responsible for the regression. Autoradiographic data using [^{3}H]thymidine labeling of dividing cells supports this interpretation. Histologic sections of biopsies 1 to 3 incubated in [^{3}H] thymidine show a diffuse pattern of labeling throughout the tumor tissue. A section of biopsy 4 shows only a small region at the edge of the tumor to contain intense labeling. These data are consistent with the hypothesis that this tumor was composed of heterogeneous subpopulations of cells. It is possible that a rapidly dividing subpopulation containing amplified c-*myc* was removed at biopsy, leaving a slower growing, regressing tumor cell subpopulation with a normal c-*myc* copy number.

The growth characteristics of radiation-induced rat skin tumors are complex and diverse. After the initial appearance of a tumor, there is usually a period of static growth, during which the tumor does not grow and remains at a small (0.1–0.2 cm^3) size. The average du-

ration of this flat static growth period was determined to be 12 ± 1 weeks for the tumors used in these experiments.

We found a significant correlation between c-*myc* gene copy number and time after tumor appearance using the composite of all tumor samples and biopsies ($p < 0.01$). The intercept of the regression line (corresponding to c-*myc* gene copy number at tumor appearance) is very close to 1. The average period required for c-*myc* gene amplification was calculated to be 14 weeks, very close to the 12-week period of the average static growth phase.

Conclusions

We conclude from our results that c-*myc* oncogene amplification is not a frequent early event in radiation-induced rat skin carcinogenesis. The data presented here argue against the idea that amplification or other alterations of the c-*myc* gene was a direct result of the exposure of target tissue to the DNA damaging effects of ionizing radiation. The evidence instead points to a role for c-*myc* in tumor progression in the rat skin tumor model.

Amplification of specific genes in cancer is probably a random event, which is why a diverse assortment of amplified genes is often found in end-stage tumors. The ability of transformed cells to amplify genes based on selective advantage (such as genes for drug resistance) can be considered to derive from the increased genetic instability associated with neoplasia. This idea derives from Nowell's hypothesis of tumor progression (*45*), wherein genetic instability and the associated increase in the frequency of gene amplification leads to the selective growth of a particular tumor cell subpopulation. Our data are consistent with the concept that amplification of c-*myc* confers a significant selective advantage on a cellular subpopulation in these tumors. Preliminary data using *in situ* hybridization support the idea of heterogeneity in c-*myc* copy number within individual cells of these tumors.

Using a statistical approach for evaluation of the data, we concluded that the average time after tumor appearance (14 weeks) required for amplification of c-*myc* is very close to the average time required for tumor growth rate to begin to increase. This is suggestive but does not prove that there is a causal relationship between the onset of rapid tumor growth and the dominant emergence of a tumor cell subpopulation containing amplified c-*myc*.

We have previously explored the role of tissue specificity in oncogene activation patterns in models of chemical carcinogenesis (*5,46,47*). The dramatic difference observed in *myc* amplification between radiation-induced skin carcinomas and sarcomas indicates that in rat skin, as in several other animal models, tumor induction by a single etiological agent often involves the participation of different molecular pathways in different tissue types (*48*). We have reported evidence for tissue-specific activation of K-*ras* in large, late-stage tumors (*11*). The activation of this gene as a function of time or progression is currently under investigation.

Although our results show that *myc* activation occurs during a late stage in tumorigenesis, it is still possible that activation of this gene is indirectly related to some effect of the radiation exposure. Studies to clarify the relationship between carcinogenic etiology and c-*myc* activation in rat and human skin tumors induced by various agents are in progress.

It is apparent that a clearer understanding of the molecular mechanisms of radiation carcinogenesis will also require systematic study of the effects of various parameters such as LET, dose, and dose rate, etc., on oncogene activation patterns. A recent report from Pellicer's laboratory showed that mouse thymomas induced by neutron irradiation exhibited a different pattern of oncogene activation than that seen with gamma radiation, including a unique *ras*-activating point mutation at codon 146 (*49*). Our laboratory is currently investigating the activation of *ras* and *myc* oncogenes in rat skin tumors induced by high LET neon ions. Radiation carcinogenesis in animals, radiation sensitivity syndromes in humans, radiation-induced transformation of cultured cells, and radioresistance are all areas in which current knowledge concerning cellular oncogenes is still somewhat fragmentary. However, a clearer picture is bound to emerge from the recently increased pace of research in these important areas of carcinogenesis.

This work was supported by grants CA 43199, CA 13343, and ES 00260 from the NIH of the Public Health Service and COO-3380 from the Department of Energy. The authors thank Betty Skocik and Tracy Ashkenazi-Kimmel for excellent technical assistance and Maureen Kohler for typing the manuscript.

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